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Research article

Bacteroides spp. and traditional fecal indicator bacteria in water quality assessment – An integrated approach for hydric resources management in urban centers

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ABSTRACT

As part of a sustainable water resources management, the Lisbon municipality identified groundwater and treated wastewater use increase as two opportunities for better and sustainable water use, with natural safeguard for public health as a priority. In this context, the aim of our research was to assess the suitability of the human-associated marker gene *Bacteroides* HF183 and the cattle feces-associated CowM2, in routine water quality monitoring as indicators for water use and reuse, providing a tool to more accurately assess public health risks. To this intent, Real-Time quantitative PCR was used for detection of human-associated marker gene *Bacteroides* HF183 and the bovine-associated CowM2, in a total of 67 samples - groundwater and wastewater at three different treatment stages of a Waste Water Treatment Plant, in Lisbon. HF183 marker gene was detected in treated and untreated wastewater samples, with significant concentration reductions from untreated (6,07 E+07 copies/mL) to secondary treated effluent (1,86 E+05 copies/mL) and a further decrease in tertiary treatment (5,74 E+04 copies/mL). In groundwater samples, this marker was also detected in concentrations ranging from 2,63 E+02 copies/mL to 2,24 E+03 copies/mL. CowM2 marker gene on the other hand was only detected in wastewater samples, with concentrations ranging from 2,47 E+02 copies/mL to 1,17 E+04 copies/mL. Our research indicates that the use of *Bacteroides* spp. in association with traditional fecal indicator bacteria (FIB) is advantageous for water managing entities in urban settings, such as Lisbon, were drainage system failures may occur. An integrated approach thus provides crucial and more adequate information towards mitigation and correction measures when fecal contamination is detected in environmental waters.

1. Introduction

1.1. Groundwater

Groundwater is a water source of particular importance as it provides for approximately half of the world's drinking water necessities (Zektser and Everett, 2004), in addition to representing an estimated 38% of the entire consumptive irrigation water use (Siebert et al., 2010). The vital importance of groundwater is rising due to ongoing unavailability of surface water resources as consequence of its contamination due to climate changes induced effects like flooding and droughts, population growth and anthropogenic activities (Rodell et al., 2018; Vörösmarty et al., 2010). However, groundwater is not hazards free, since this resource can also be affected by factors including pollution which infiltrates in the soils, excessive pumping of the groundwater or its salinization, reasons why more demanding management and monitoring are being required on a local and global scale (de Graaf et al., 2019). Lisbon municipality has several groundwater resources identified and available, but while some are used and monitored, others with significant potential remain underexploited, as they are still not adequately

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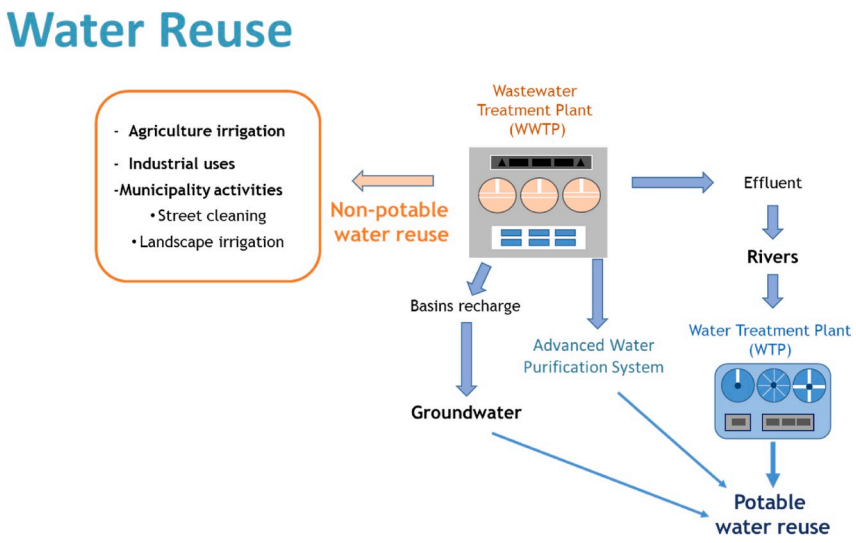

Fig. 1. Current possible uses for treated wastewater.

characterized from a microbiological and chemical standpoint.

1.2. Recycled water

Positioned in an advanced stage of the water cycle is treated wastewater. It is currently used worldwide as an alternative for numerous purposes such as agricultural and landscape irrigation, industrial processes, aquifer recharge, street cleaning or even possible potable use (Fig. 1). The demand for recycled water will continue to increase and play a crucial role in hydric resources management and future urban planning (Angelakis et al., 2018). To this intent, Waste Water Treatment Plants (WWTPs) are designed to reduce pathogen loads in order to avoid public health risks, usually monitored via fecal indicator bacteria (FIB) as surrogates for pathogens. Several studies however have been reporting incomplete pathogen removal (Blatchley et al., 2007; El-Senousy et al., 2007; Lazarova et al., 2001; Sano et al., 2016).

1.3. Fecal indicator bacteria (FIB)

Routine water quality assessment has been focused fundamentally on detecting FIB, usually *Escherichia coli* (*E. coli*) and *Enterococcus* spp. (EPA, 2012; José Figueras and Borrego, 2010; Leclerc et al., 2001; Saxena et al., 2015; Tallon et al., 2005; WHO, 2003), even though its presence is not always well correlated with pathogen existence (Arnold et al., 2013; Bonadonna et al., 2002; Bradshaw et al., 2016; Harwood et al., 2005; Hörman et al., 2004; Lemarchand and Lebaron, 2003; Lund, 1996; McQuaig et al., 2012; Zhang et al., 2016). Another FIB limitation is a result of factors like the ability for FIB not only to survive, but to possibly grow in several environments, including sands, soils, aquatic vegetation or sediments (Anderson et al., 2005; Badgley et al., 2011; Bradshaw et al., 2016; Ishii et al., 2006; Solo-Gabriele et al., 2000; Zhang et al., 2015), potentially over estimating FIB presence.

In addition, with FIB being excreted in the feces of all warm-blooded animals (Field and Samadpour, 2007; Harwood et al., 1999; Leclerc et al., 2001), current prevailing water quality indicators also fail to provide information regarding the source of contamination, which impairs mitigation and remediation actions, as well as accurate risk assessment (Fujioka et al., 2015; Soller et al., 2010). To overcome this gap, microbial source tracking (MST) tools targeting host-associated molecular marker genes have been developed, aiming to distinguish the different sources of fecal contamination, from human to several animals, including dog, chicken, cattle, pig, seagull, possum, duck, horse (Ahmed et al., 2016a; Harwood et al., 2014), and also virus, bacteria or protozoa (Bernhard and Field, 2000; Scott et al., 2005; Ufnar et al., 2006).

1.4. Bacteroides spp.

Within bacterial targets, *Bacteroides* markers are an attractive option, due to several characteristics such as a short survival rates outside the host, inability to multiply in the environment, are exclusive to warm-blooded animals and constituents of a larger portion of fecal bacteria when compared to FIB (Bernhard and Field, 2000; Kreader, 1995; Sghir et al., 2000). Human associated *Bacteroides* marker HF183 has been studied extensively and exhibits several important characteristics as a MST marker, as it presents an overall high host sensitivity and host specificity (Ahmed et al., 2016a, 2012), besides concentrations in human feces with little or no temporal and geographical variations (Ahmed et al., 2016a, 2016b). Since human fecal contamination poses greater risks to human health, numerous MST studies focus only in this source, due to its considered greater importance by regulators and management authorities (Scott et al., 2002; Soller et al., 2010; Wade et al., 2003). Nonetheless, several other host-associated molecular marker genes have been developed to identify animal sources of fecal contamination, including the cattle feces-associated CowM2 (Shanks et al., 2008).

1.5. Lisbon Municipality

In Lisbon, a city with over half a million inhabitants (INE, 2018), municipal services accounted for about 32% of the cities' non domestic potable water consumption in 2014, mostly attributable to landscape irrigation and street cleaning (Lisboa E-Nova, 2014). In a continuous effort, this value was significantly lowered to 18% in 2018 (Lisboa E-Nova, 2018). The main drinking water supply of Lisbon originates about 140 km from the city and it also comprises several treatment processes and continuous monitoring equipment, aimed at ensuring the highest quality of water supplied (Lisboa E-Nova, 2014). Therefore, a sustainable water resources management requires looking for alternatives water supplies, particularly aiming non-potable uses. The city of Lisbon, which recently attained the 2020 European Green Capital award, is committed in turning environmental challenges into opportunities, by means of ensuring the best environmental management - from urban planning to maintaining a sustainable hydric resources management. To this concern, two opportunities for better and sustainable water use have been identified: increase the amount of groundwater and treated wastewater use for municipal services, not disregarding safeguard for public health as a priority.

Table 1
Primers and probes for the HF183/BacR207 and CowM2 Real-Time qPCR assays.

Marker genes	Primers (F,R) and probes (P)	Reference
HF183	F: ATC ATG AGT TCA CAT GTC CG R: CTT CCT CTC AGA ACC CCT ATC C P: FAM-CTA ATG GAA CGC ATC CC-MGBEQ	Green et al. (2014)
CowM2	F: CGG CCA AAT ACT CCT GAT CGT R: GCT TGT TGC GTT CCT TGA GAT AAT P: FAM – AGG CAC CTA TGT CCT TTA CCT CAT CAA CTA CAG ACA –MGBEQ	Shanks et al. (2008)

In this context, the aim of our research was to assess the suitability of the human-associated marker gene *Bacteroides* HF183 (Green et al., 2014) and the cattle feces-associated CowM2 (Shanks et al., 2008) as indicators for water use and reuse in routine water quality monitoring, thus providing a MST tool to more accurately assess public health risks. Importantly, we also aimed to assess the usefulness of detecting *Bacteroides* spp. combined with FIB and enteric viruses, relating to our previously published work (Teixeira et al., 2020).

2. Materials and methods

2.1. Sampling

Field work was carried out from February-2018 to December-2018 with a total of 67 samples collected. Sampling included groundwater (GW1, GW2) samples ($n = 19$) collected at two different sites in Lisbon and wastewater collected at a WWTP in the Lisbon district at three different stages – untreated influent (WW1) ($n = 15$), effluent with secondary treatment (WW2) ($n = 15$) and effluent with tertiary, sand filtration and UV treatment (WW3) ($n = 15$). Blank assays (BR) were also performed with distilled, sterilized water samples ($n = 3$). All samples were collected in sterile polyethylene containers and stored at 4 °C for less than 2 h until chemical and microbiological determinations were initiated. Time lapse between sample collection and laboratorial processing did not exceed 24 h.

2.2. HF183 and CowM2 Real-Time qPCR assays

Briefly, aliquots of 25 mL (untreated wastewater samples) and 100 mL (blank assay, secondary and tertiary treated wastewater and groundwater samples) were filtered through 0.22 μm pore size nitrocellulose membranes (Merck Millipore, USA). Selected volumes for filtration were based on previous studies targeting HF183 (Green et al., 2014) and CowM2 (Shanks et al., 2006). DNA was extracted using Aquadien™ DNA Extraction and Purification Kit according to manufacturer's instructions and stored at −20 °C until analysis. The concentration of DNA in each sample was measured in a Nano-Drop 2000 instrument (Thermo Scientific, MA, USA) and then adjusted to 10 ng/μL. For the HF183/Bac287 Real-Time qPCR assay, 10 μL reaction mixtures containing 1x SsoAdvanced Universal Probe Supermix (Biorad, France), 0.2 mg/mL bovine serum, 0.5 μM of each primer, 80 nM (FAM)-labeled probe (Table 1) and 2 μL of DNA template or molecular-grade water (no-template control - NTC) were prepared. The thermal cycling conditions were 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C (Green et al., 2014). For the CowM2 qPCR assay, 10 μL reaction mixtures containing 1x SsoAdvanced Univ Probes Supermix (Biorad, France), 0.2 mg/mL bovine serum, 1 μM of each primer, 80 nM (FAM)-labeled probe and 2 μL of DNA template or molecular-grade water (NTC) were prepared. The thermal cycling conditions were 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C (Shanks et al., 2008). For both qPCR assays, synthetic DNA fragments containing 133 bp (for HF183) and 93 bp (for CowM2) were purchased from Eurofins Genomics (Ebersberg, Germany) and Real-Time qPCR standards from the synthetic DNA were prepared, ranging from 1,00 E+02 to 1,00 E+05 copies/μL of DNA. The Real-Time qPCR assays were performed using a Rotor-Gene Q thermal cycler (QIAGEN Hilden, Germany). All Real-Time qPCR reactions were performed in triplicate.

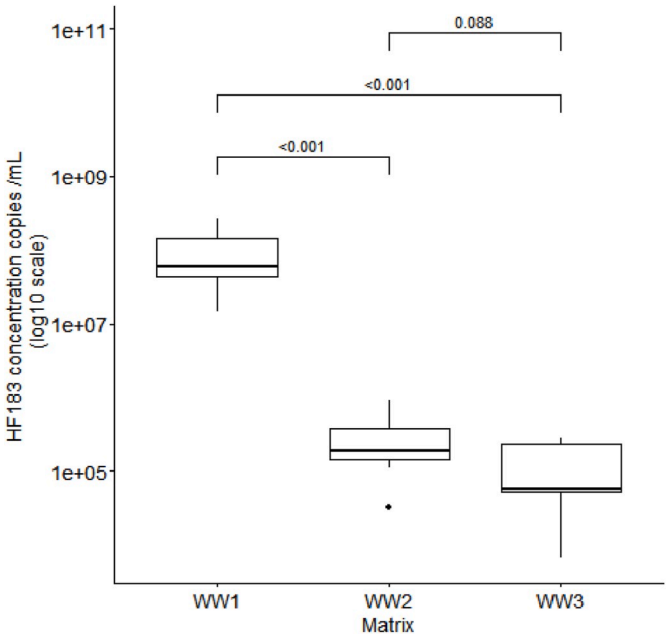

Fig. 2. Boxplot of concentration values (copies/mL) for HF183 in wastewaters (median of 6,07 E+07, 1,86 E+05 and 5,74 E+04 in WW1, WW2 and WW3, respectively). *p* values of multiple comparisons with Bonferroni correction are indicated on top of the boxplots.

2.3. Enteric viruses, microbiological, physical and chemical assessment

As part of this project, total coliforms, *Escherichia coli*, enterococci, fecal coliforms, heterotrophic plate count at 22 °C and 37 °C, temperature, pH, free chlorine, total organic carbon, conductivity and chlorides, and also the presence of enteric viruses was previously assessed, as described elsewhere (Teixeira et al., 2020).

2.4. Statistical analysis

A descriptive analysis was made for numeric variables. For comparing different measures among the three wastewater treatment phases, a Kruskal-Wallis non-parametric test was used. When differences were found, a multiple comparison with Bonferroni correction was also performed. A significance level of 5% was considered and the statistical analysis was performed using statistical software R, version 3.4.3. (R Core Team, 2017).

3. Results

HF183 marker gene was identified in every untreated wastewater influent ($n = 15$), effluent with secondary treatment ($n = 15$) and effluent with tertiary treatment ($n = 15$) samples. The median value of HF183 concentration decreased from WW1 to WW2 and WW3 (6,07 E+07 copies/mL to 1,86 E+05 copies/mL and 5,74 E+04 copies/mL) where statistically significant differences between these distributions were found ($p < 0.001$). Performing multiple comparisons with Bonferroni correction statistically significant differences were found between WW1 and the two other matrix, but not between WW2 and WW3, although a decrease was observed between the WW2 and WW3 HF183 counts (Fig. 2).

HF183 gene marker was also detected in samples from groundwater origin, with concentrations ranging from 2,63 E+02 copies/mL to 2,24

Table 2
Total samples analyzed and median concentrations for positive samples (copies/mL) for the HF183 marker in blank assays (BR) and groundwater (GW1, GW2). LOD = Limit of Detection.

		Sample Matrix		
Marker		BR	GW1	GW2
HF183	Total samples analyzed	3	8	11
	Median concentration for positive samples (copies/mL)	<LOD	3,40 E+02 (n = 4)	2,24 E+03 (n = 1)

E+03 copies/mL. None of the blank assays exhibited positive results for this marker (Table 2).

CowM2 marker gene on the other hand was only detected in a small number of secondary treated and untreated wastewater samples, with concentrations ranging from 2,47 E+02 copies/mL to 1,17 E+04 copies/mL. None of the groundwater or blank assays samples exhibited quantifiable results for this marker (Table 3).

4. Discussion

4.1. Groundwater

The characteristically high concentration of the HF183 marker in untreated wastewater facilitates the detection of sewage contamination in recreational and environmental waters (Hughes et al., 2017). In our previous work (Teixeira et al., 2020), a low overall contamination level of fecal origin was observed at the sampled groundwater sites and, based on FIB alone, the obtained results for these groundwater samples are in compliance with national legislation, deeming them suitable for non-potable uses, e.g. irrigation. However, in this study several samples tested positive for the presence of human-associated marker gene HF183 pointing to a sewage contamination, most probably originating from a drainage system failure, since these sites are located in urban areas. Considering the current uses for these waters, namely landscape and crops irrigation, the possible pathogen presence linked to human wastewater may represent a public health risk, this is the reason why origin of the contamination should always be identified to allow mitigation of the contamination problem. FIB role as indicators for water quality assessment remains undoubtedly of great usefulness but, in cases where fecal contamination is detected in environmental waters, it seems to fall short in providing the necessary information for a realistic health risk assessment. For groundwater quality assessment, we propose the use of FIB in association with the MST marker gene HF183, since it is particularly useful in urban areas such as Lisbon, were drainage system failures may occur and contaminate the groundwater sources. Even though CowM2 marker gene was undetected in the targeted groundwater sites, water managing entities located in rural areas may probably also find it useful for exploring other sources of animal fecal contamination in association with FIB, such as the one targeted in this study – CowM2. Some studies have however reported occasional false-positives concerning the HF183 marker (Ballesté et al., 2010; Gawler et al., 2007; Shanks et al., 2010), therefore it has been suggested (Green et al., 2014) to combine additional assays in order to decrease the possibility of overestimating human contaminants in areas with animal sources of fecal contamination, like dogs or chickens (Layton et al., 2013). To some extent, it can be difficult to define an assay as exclusively human

Table 3
Total samples analyzed and median concentrations for positive samples (copies/mL) for the CowM2 marker in blank assays (BR), groundwater (GW1, GW2), untreated (WW1), secondary (WW2) and tertiary treated wastewater (WW3). LOD = Limit of Detection.

		Sample Matrix					
Marker		BR	GW1	GW2	WW1	WW2	WW3
Cow M2	Total samples analyzed	3	8	11	15	15	15
	Median Concentration for positive samples (copies/mL)	<LOD	<LOD	<LOD	1,01 E+04 (n = 3)	2,47 E+02 (n = 1)	<LOD

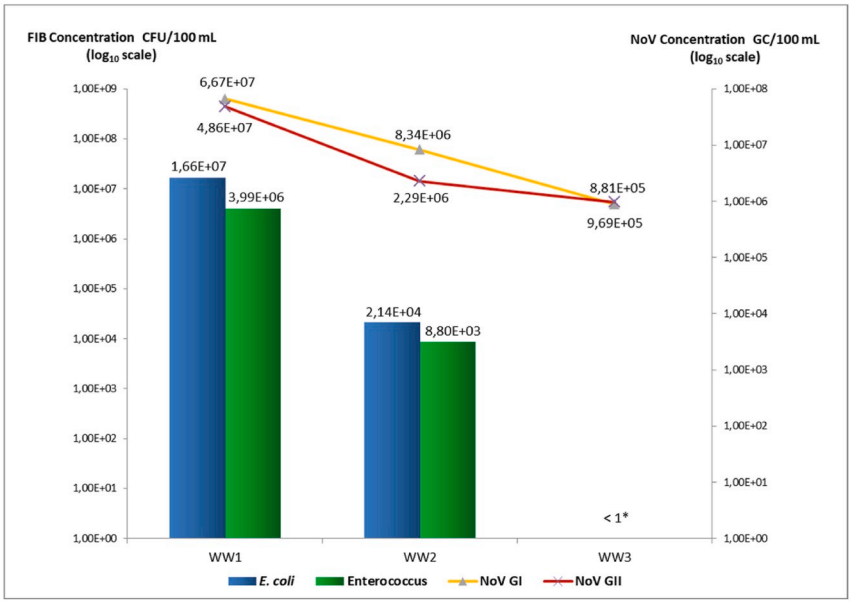

Fig. 3. Median concentrations for positive samples in untreated/secondary treated/tertiary treated wastewater samples (WW1/WW2/WW3) for *E. coli* (n = 15/15/0), Enterococcus (n = 15/15/0), NoV GI (n = 15/7/5) and NoV GII (n = 15/11/3). *undetected in a total of n = 15 samples. CFU – Colony Forming Units. GC – Genomic Copies. Adapted from original data presented in (Teixeira et al., 2020).

specific, given the reported animal cross-reactions (Feng and McLellan, 2019).

4.2. Wastewater

WWTP effectiveness in pathogen removal is critical as wastewater may represent a potential route of exposure to a wide array of pathogens including viruses, protozoa, bacteria or fungi, particularly given the fact that wastewater treatment processes may not completely remove pathogens, even considering tertiary treatment (Blatchley et al., 2007; El-Senousy et al., 2007; Lazarova et al., 2001; Sano et al., 2016). Our results show that the presence of HF183 marker in treated and untreated wastewaters samples, with results comparable to previous studies targeting this marker gene (Ahmed et al., 2016a; Chern et al., 2014). Notably, and in association with our previous work (Teixeira et al., 2020), HF183 marker gene concentration reductions in WWTP treatment stages appear to display a similar behavior as Norovirus (NoV) genogroups I (GI) and II (GII) (Fig. 3), suggesting a potential role for this marker in monitoring wastewater treatment efficiency. We propose that future studies should be performed in order to validate this possible role, and also to assess the possible existing correlations between this marker and NoV. Considering wastewaters with UV tertiary treatment, where we detected HF183 marker (median 5,74 E+04 copies/mL) although FIB were removed, we may speculate that the *Bacteroides* detected in this treatment stage were probably dead but, nevertheless, the detection of the HF183 DNA marker is still possible. This hypothesis could be clarified by looking for viable *Bacteroides* in tertiary treatment samples in future studies.

The reduced number of samples ($n = 4$) testing positive for the CowM2 marker in treated and untreated wastewaters does not allow to draw significant conclusions to our study, except that there is also some bovine associated contamination in the urban context studied – Lisbon.

5. Conclusions

Groundwater and treated wastewater enhanced use is crucial for a sustainable hydric resources management, which implies a continuous search for the best practices in water quality assessment. With our research, we can conclude that an integrated approach for water quality monitoring, comprising the use of HF183 gene marker associated with FIB detection provides crucial and more adequate information towards mitigation and correction measures when fecal contamination in groundwater is detected in urban sites, were drainage system failures may occur. Moreover, the use of the HF183 gene marker may also have a future role in evaluating wastewater treatment efficiency in WWTPs, as it appears to display a similar behavior as NoV GI/GII, particularly in samples were FIB were completely removed. Detection of *Bacteroides* spp. markers in WWTPs can be a useful tool not only monitor wastewater treatment efficiency in a rapid manner, but also to characterize untreated influent concerning concentrations and identify the sources of fecal contamination.

Declaration of competing interest

There are no conflicts of interest to declare.

CRediT authorship contribution statement

Pedro Teixeira: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Project administration, Funding acquisition. **Deodália Dias:** Writing - review & editing. **Sílvia Costa:** Investigation. **Bárbara Brown:** Investigation. **Susana Silva:** Formal analysis, Visualization, Writing - review & editing. **Elisabete Valério:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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References

Ahmed, W., Masters, N., Toze, S., 2012. Consistency in the host specificity and host sensitivity of the Bacteroides HF183 marker for sewage pollution tracking. Lett. Appl. Microbiol. 55, 283–289. https://doi.org/10.1111/j.1472-765X.2012.03291.x.

Ahmed, W., Hughes, B., Harwood, V.J., 2016a. Current status of marker genes of bacteroides and related taxa for identifying sewage pollution in environmental waters. Water 8, 231. https://doi.org/10.3390/w8060231.

Ahmed, W., Sidhu, J.P.S., Smith, K., Beale, D.J., Gyawali, P., Tozea, S., 2016b. Distributions of fecal markers in wastewater from different climatic zones for human fecal pollution tracking in Australian surface waters. Appl. Environ. Microbiol. 82, 1316–1323. https://doi.org/10.1128/AEM.03765-15.

Anderson, K.L., Whitlock, J.E., Valerie, J., Harwood, V.J., 2005. Persistence and differential survival of fecal indicator bacteria in subtropical waters and sediments. Appl. Environ. Microbiol. 71, 3041–3048. https://doi.org/10.1128/AEM.71.6.3041-3048.2005.

Angelakis, A.N., Asano, T., Bahri, A., Jimenez, B.E., Tchobanoglous, G., 2018. Water reuse: from ancient to modern times and the future. Front. Environ. Sci. 6, 26. https://doi.org/10.3389/fenvs.2018.00026.

Arnold, B.F., Schiff, K.C., Griffith, J.F., Gruber, J.S., Yau, V., Wright, C.C., Wade, T.J., Burns, S., Hayes, J.M., Mcgee, C., Gold, M., Cao, Y., Weisberg, S.B., Colford, J.M., 2013. Swimmer illness associated with marine water exposure and water quality indicators: impact of widely used assumptions. Epidemiology 24, 845–853. https://doi.org/10.1097/01.ede.0000434431.06765.4a.

Badgley, B.D., Thomas, F.I.M., Harwood, V.J., 2011. Quantifying environmental reservoirs of fecal indicator bacteria associated with sediment and submerged aquatic vegetation. Environ. Microbiol. 13, 932–942. https://doi.org/10.1111/j.1462-2920.2010.02397.x.

Ballesté, E., Bonjoch, X., Belanche, L.A., Blanch, A.R., 2010. Molecular indicators used in the development of predictive models for microbial source tracking. Appl. Environ. Microbiol. 76, 1789–1795. https://doi.org/10.1128/AEM.02350-09.

Bernhard, A.E., Field, K.G., 2000. Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes. Appl. Environ. Microbiol. 66, 1587–1594. https://doi.org/10.1128/AEM.66.4.1587-1594.2000.

Blatchley, E.R., Gong, W.-L., Alleman, J.E., Rose, J.B., Huffman, D.E., Otaki, M., Lisle, J. T., 2007. Effects of wastewater disinfection on waterborne bacteria and viruses. Water Environ. Res. 79, 81–92. https://doi.org/10.2175/106143006x102024.

Bonadonna, L., Briancesco, R., Ottaviani, M., Veschetti, E., 2002. Occurrence of Cryptosporidium oocysts in sewage effluents and correlation with microbial, chemical and physical water variables. Environ. Monit. Assess. 75, 241–252. https://doi.org/10.1023/A:1014852201424.

Bradshaw, J.K., Snyder, B.J., Oladeinde, A., Spidle, D., Berrang, M.E., Meinersmann, R.J., Oakley, B., Sidle, R.C., Sullivan, K., Molina, M., 2016. Characterizing relationships among fecal indicator bacteria, microbial source tracking markers, and associated waterborne pathogen occurrence in stream water and sediments in a mixed land use watershed. Water Res. 101, 498–509. https://doi.org/10.1016/j.watres.2016.05.014.

Chern, E.C., Brenner, K., Wymer, L., Haugland, R.A., 2014. Influence of wastewater disinfection on densities of culturable fecal indicator bacteria and genetic markers. J. Water Health 12, 410–417. https://doi.org/10.2166/wh.2013.179.

de Graaf, I.E.M., Gleeson, T., Rens, van Beek, L.P.H., Sutanudjaja, E.H., Bierkens, M.F.P., 2019. Environmental flow limits to global groundwater pumping. Nature 574, 90–94. https://doi.org/10.1038/s41586-019-1594-4.

E-Nova, Lisboa, 2014. Matriz da Água de Lisboa 2014 44.

E-Nova, Lisboa, 2018. Grandes Números da Água [WWW Document]. Obs. Água (accessed 1.14.20). http://www.observatorios-lisboa.pt/agua.html.

El-Senousy, W.M., Guix, S., Abid, I., Pintó, R.M., Bosch, A., 2007. Removal of astrovirus from water and sewage treatment plants, evaluated by a competitive reverse transcription-PCR. Appl. Environ. Microbiol. 73, 164–167. https://doi.org/10.1128/AEM.01748-06.

EPA, 2012. Recreational Water Quality Criteria. United States Environ, pp. 1–69. Prot. Agency.

Feng, S., McLellan, S.L., 2019. Highly specific sewage-derived bacteroides quantitative PCR assays target sewage-polluted waters. Appl. Environ. Microbiol. https://doi.org/10.1128/AEM.02696-18.

Field, K.G., Samadpour, M., 2007. Fecal source tracking, the indicator paradigm, and managing water quality. Water Res. 41, 3517–3538. https://doi.org/10.1016/j.watres.2007.06.056.

Fujioka, R.S., Solo-Gabriele, H.M., Byappanahalli, M.N., Kirs, M., 2015. U.S. recreational water quality criteria: a vision for the future. Int. J. Environ. Res. Publ. Health 12, 7752–7776. https://doi.org/10.3390/ijerph120707752.

Gawler, A.H., Beecher, J.E., Brandão, J., Carroll, N.M., Falcão, L., Gourmelon, M., Masterson, B., Nunes, B., Porter, J., Rincé, A., Rodrigues, R., Thorp, M., Martin Walters, J., Meijer, W.G., 2007. Validation of host-specific Bacteriodales 16S rRNA genes as markers to determine the origin of faecal pollution in Atlantic Rim countries of the European Union. Water Res. 41, 3780–3784. https://doi.org/10.1016/j.watres.2007.01.028.

Green, H.C., Haugland, R.A., Varma, M., Millen, H.T., Borchardt, M.A., Field, K.G., Walters, W.A., Knight, R., Sivaganesan, M., Kelty, C.A., Shanks, O.C., 2014. Improved HF183 quantitative real-time PCR assay for characterization of human fecal pollution in ambient surface water samples. Appl. Environ. Microbiol. 80, 3086–3094. https://doi.org/10.1128/AEM.04137-13.

Harwood, V.J., Butler, J., Parrish, D., Wagner, V., 1999. Isolation of fecal coliform bacteria from the diamondback terrapin (Malaclemys terrapin centrata). Appl. Environ. Microbiol. 65, 865–867.

Harwood, V.J., Levine, A.D., Scott, T.M., Chivukula, V., Lukasik, J., Farrah, S.R., Rose, J. B., 2005. Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. Appl. Environ. Microbiol. 71, 3163–3170. https://doi.org/10.1128/AEM.71.6.3163.

Harwood, V.J., Staley, C., Badgley, B.D., Borges, K., Korajkic, A., 2014. Microbial source tracking markers for detection of fecal contamination in environmental waters: relationships between pathogens and human health outcomes. FEMS Microbiol. Rev. 38, 1–40. https://doi.org/10.1111/1574-6976.12031.

Hörman, A., Rimhanen-finne, R., Maunula, L., Bonsdorff, C. Von, Torvela, N., 2004. Indicator organisms in surface water in and indicator organisms in surface water in southwestern Finland, 2000-2001. Appl. Environ. Microbiol. 70, 87–95. https://doi.org/10.1128/AEM.70.1.87-95.2004.

Hughes, B., Beale, D.J., Dennis, P.G., Cook, S., Ahmed, W., 2017. Cross-comparison of human wastewater-associated molecular markers in relation to fecal indicator bacteria and enteric viruses in recreational beach waters. Appl. Environ. Microbiol. 83, e00028-17 https://doi.org/10.1128/AEM.00028-17.

INE, 2018. População residente: total e por grandes grupos etários [WWW Document]. PORDATA (accessed 1.27.20). https://www.pordata.pt/Municipios/População+residente+total+e+por+grandes+grupos+etários-390.

Ishii, S., Yan, T., Shively, D.A., Byappanahalli, M.N., Whitman, R.L., Sadowsky, M.J., 2006. Cladophora (Chlorophyta) spp. harbor human bacterial pathogens in nearshore water of Lake Michigan. Appl. Environ. Microbiol. 72, 4545–4553. https://doi.org/10.1128/AEM.00131-06.

José Figueras, M., Borrego, J.J., 2010. New perspectives in monitoring drinking water microbial quality. Int. J. Environ. Res. Publ. Health 7, 4179–4202. https://doi.org/10.3390/ijerph7124179.

Kreader, C.A., 1995. Design and evaluation of Bacteroides DNA probes for the specific detection of human fecal pollution. Appl. Environ. Microbiol. 61, 1171–1179.

Layton, B.A., Cao, Y., Ebentier, D.L., Hanley, K., Balleste, E., Brandão, J., Byappanahalli, M., Converse, R., Farnleitner, A.H., Gentry-Shields, J., Gidley, M.L., Gourmelon, M., Lee, C.S., Lee, J., Lozach, S., Madi, T., Meijer, W.G., Noble, R., Peed, L., Reischer, G.H., Rodrigues, R., Rose, J.B., Schriewer, A., Sinigalliano, C., Srinivasan, S., Stewart, J., Van De Werfhorst, L.C., Wang, D., Whitman, R., Wuertz, S., Jay, J., Holden, P.A., Boehm, A.B., Shanks, O., Griffith, J.F., 2013. Performance of human fecal anaerobe-associated PCR-based assays in a multi-laboratory method evaluation study. Water Res. 47, 6897–6908. https://doi.org/10.1016/j.watres.2013.05.060.

Lazarova, V., Levine, B., Sack, J., Cirelli, G., Jeffrey, P., Muntau, H., Salgot, M., Brissaud, F., 2001. Role of water reuse for enhancing integrated water management in Europe and Mediterranean countries. Water Sci. Technol. 43, 25–33. https://doi.org/10.2166/wst.2001.0571.

Leclerc, H., Mossel, D.A.A., Edberg, S.C., Struijk, C.B., 2001. Advances in the bacteriology of the coliform group: their suitability as markers of microbial water safety. Annu. Rev. Microbiol. 55, 201–234. https://doi.org/10.1146/annurev.micro.55.1.201.

Lemarchand, K., Lebaron, P., 2003. Occurrence of Salmonella spp. and Cryptosporidium spp. in a French coastal watershed: relationship with fecal indicators. FEMS Microbiol. Lett. 218, 203–209. https://doi.org/10.1016/S0378-1097(02)01135-7.

Lund, V., 1996. Evaluation of E. Coli as an indicator for the presence of Campylobacter jejuni and Yersinia enterocolitica in chlorinated and untreated oligotrophic lake water. Water Res. 30, 1528–1534. https://doi.org/10.1016/0043-1354(96)00034-6.

McQuaig, S., Griffith, J., Harwood, V.J., 2012. Association of fecal indicator bacteria with human viruses and microbial source tracking markers at coastal beaches impacted by nonpoint source pollution. Appl. Environ. Microbiol. 78, 6423–6432. https://doi.org/10.1128/AEM.00024-12.

R Core Team, 2017. R: A Language and Environment for Statistical Computing [WWW Document]. R A Lang. Environ. Stat. Comput (accessed 7.16.19). https://www.r-project.org/.

Rodell, M., Famiglietti, J.S., Wiese, D.N., Reager, J.T., Beaudoing, H.K., Landerer, F.W., Lo, M.H., 2018. Emerging trends in global freshwater availability. Nature 557, 651–659. https://doi.org/10.1038/s41586-018-0123-1.

Sano, D., Amarasiri, M., Hata, A., Watanabe, T., Katayama, H., 2016. Risk management of viral infectious diseases in wastewater reclamation and reuse: Review. Environ. Int. 91, 220–229. https://doi.org/10.1016/j.envint.2016.03.001.

Saxena, G., Bharagava, R.N., Kaithwas, G., Raj, A., 2015. Microbial indicators, pathogens and methods for their monitoring in water environment. J. Water Health 13, 319–339. https://doi.org/10.2166/wh.2014.275.

Scott, T.M., Rose, J.B., Jenkins, T.M., Farrah, S.R., Lukasik, J., 2002. Microbial source tracking: current methodology and future directions. Appl. Environ. Microbiol. 68, 5796–5803. https://doi.org/10.1128/AEM.68.12.5796-5803.2002.

Scott, T.M., Jenkins, T.M., Lukasik, J., Rose, J.B., 2005. Potential use of a host associated molecular marker in Enterococcus faecium as an index of human fecal pollution. Environ. Sci. Technol. 39, 283–287. https://doi.org/10.1021/es035267n.

Sghir, A., Gramet, G., Suau, A., Rochet, V., Pochart, P., Dore, J., 2000. Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. Appl. Environ. Microbiol. 66, 2263–2266. https://doi.org/10.1128/AEM.66.5.2263-2266.2000.

Shanks, O.C., Santo Domingo, J.W., Lamendella, R., Kelty, C.A., Graham, J.E., 2006. Competitive metagenomic DNA hybridization identifies host-specific microbial genetic markers in cow fecal samples. Appl. Environ. Microbiol. 72, 4054–4060. https://doi.org/10.1128/AEM.00023-06.

Shanks, O.C., Atikovic, E., Blackwood, A.D., Lu, J., Noble, R.T., Domingo, J.S., Seifring, S., Sivaganesan, M., Haugland, R.A., 2008. Quantitative PCR for detection and enumeration of genetic markers of bovine fecal pollution. Appl. Environ. Microbiol. 74, 745–752. https://doi.org/10.1128/AEM.01843-07.

Shanks, O.C., White, K., Kelty, C.A., Sivaganesan, M., Blannon, J., Meckes, M., Varma, M., Haugland, R.A., 2010. Performance of PCR-based assays targeting Bacteroidales genetic markers of human fecal pollution in sewage and fecal samples. Environ. Sci. Technol. 44, 6281–6288. https://doi.org/10.1021/es100311n.

Siebert, S., Burke, J., Faures, J.M., Frenken, K., Hoogeveen, J., Döll, P., Portmann, F.T., 2010. Groundwater use for irrigation - a global inventory. Hydrol. Earth Syst. Sci. 14, 1863–1880. https://doi.org/10.5194/hess-14-1863-2010.

Soller, J.A., Schoen, M.E., Bartrand, T., Ravenscroft, J.E., Ashbolt, N.J., 2010. Estimated human health risks from exposure to recreational waters impacted by human and non-human sources of faecal contamination. Water Res. 44, 4674–4691. https://doi.org/10.1016/j.watres.2010.06.049.

Solo-Gabriele, H.M., Wolfert, M.A., Desmarais, T.R., Palmer, C.J., 2000. Sources of Escherichia coli in a coastal subtropical environment. Appl. Environ. Microbiol. 66, 230–237. https://doi.org/10.1128/AEM.66.1.230-237.2000.

Tallon, P., Magajna, B., Lofranco, C., Leung, K.T., 2005. Microbial indicators of faecal contamination in water: a current perspective. Water. Air. Soil Pollut 166, 139–166. https://doi.org/10.1007/s11270-005-7905-4.

Teixeira, P., Costa, S., Brown, B., Silva, S., Rodrigues, R., Valério, E., 2020. Quantitative PCR detection of enteric viruses in wastewater and EnvironmentalWater sources by the Lisbon municipality: a case study. Water (Switzerland) 12, 544. https://doi.org/10.3390/w12020544.

Ufnar, J.A., Wang, S.Y., Christiansen, J.M., Yampara-Iquise, H., Carson, C.A., Ellender, R. D., 2006. Detection of the nifH gene of Methanobrevibacter smithii: a potential tool to identify sewage pollution in recreational waters. J. Appl. Microbiol. 101, 44–52. https://doi.org/10.1111/j.1365-2672.2006.02989.x.

Vörösmarty, C.J., McIntyre, P.B., Gessner, M.O., Dudgeon, D., Prusevich, A., Green, P., Glidden, S., Bunn, S.E., Sullivan, C.A., Liermann, C.R., Davies, P.M., 2010. Global threats to human water security and river biodiversity. Nature 467, 555–561. https://doi.org/10.1038/nature09440.

Wade, T.J., Pai, N., Eisenberg, J.N.S., Colford Jr., J.M., 2003. Do U.S. Environmental Protection Agency water quality guidelines for recreational waters prevent gastrointestinal illness? A systematic review and meta-analysis. Environ. Health Perspect. 111, 1102–1109.

WHO, 2003. Guidelines for safe recreational water environments - coastal and Fresh waters. new Guidelines for Safe Recreational Water 1, 253.

Zektser, I.S., Everett, L.G., 2004. Groundwater Resources of the World and Their Use. IhP Series on groundwater. No. 6.

Zhang, Q., He, X., Yan, T., 2015. Impact of indigenous microbiota of subtidal sand on fecal indicator bacteria decay in beach systems: a microcosm study. Environ. Sci. Water Res. Technol. 1, 306–315. https://doi.org/10.1039/c5ew00004a.

Zhang, Q., Eichmiller, J.J., Staley, C., Sadowsky, M.J., Ishii, S., 2016. Correlations between pathogen concentration and fecal indicator marker genes in beach environments. Sci. Total Environ. 15, 826–830. https://doi.org/10.1016/j.scitotenv.2016.08.122.